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Insulin Binding to Erythrocytes from Pregnant, Postpartum, Follicular and Luteal States

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Summary: Specific binding of [^{125}I]insulin to isolated erythrocytes from four groups of women was investigated:

(A) pregnant subjects between weeks 38 and 40 of pregnancy (n = 18),

(B) postpartum subjects within 6 days after delivery (n = 20),

(C) normal women during the follicular phase of the menstrual cycle (n = 12) and

(D) normal women during the luteal phase of the menstrual cycle (n = 11).

Specific [^{125}I]insulin binding (fraction), fasting plasma glucose concentrations (mmol/l) and the corresponding insulin concentrations (mU/l) were

0.074 ± 0.012 / 4.00 ± 0.58 / 29.4 ± 21.4 for group A,
0.065 ± 0.016 / 4.40 ± 0.75 / 41.5 ± 26.2 for group B,
0.052 ± 0.008 / 4.58 ± 0.62 / 6.7 ± 4.0 for group C and
0.054 ± 0.011 / 4.49 ± 0.63 / 8.3 ± 5.9 for group D.

By using a modified *Scatchard* analysis, statistically significant differences were observed between the receptor affinities of the groups A and D, B and D, A and C.

The receptor affinities and concentrations were not significantly different between the follicular and the luteal phases. From the data, no inverse correlation between the plasma insulin concentration and receptor binding was seen, i.e. the phenomenon of downregulation of insulin receptor concentration with hyperinsulinaemia seemed not to apply to erythrocytes.

The present results suggest that insulin binding to erythrocytes is modulated preferably or even exclusively by an alteration of receptor affinity and that short-term changes in insulin binding to erythrocytes are not caused by an alteration of receptor concentration.

Vergleich der Bindung von Insulin an Erythrocyten während und nach Schwangerschaft und in der Proliferations- und der Sekretionsphase des Menstruations-Cyclus

Zusammenfassung: Die Bindung von [^{125}I]Insulin an Erythrocyten wird bei vier Gruppen von Frauen untersucht:

A Schwangere in der 38.–40. Schwangerschaftswoche (n = 18),
B Frauen innerhalb von 6 Tagen nach Entbindung (n = 20),
C Frauen in der follikulären Cyclusphase (n = 12) und
D Frauen in der lutealen Cyclusphase (n = 11).

Die spezifische Bindung des [^{125}I]Insulins (Anteil), die Plasma-Glucose-Konzentration (mmol/l) und die zugehörige Insulinkonzentration (mU/l) sind für die vier Gruppen:

A (0,074 ± 0,012 / 4,00 ± 0,58 / 29,4 ± 21,4),
B (0,065 ± 0,016 / 4,40 ± 0,75 / 41,5 ± 26,2),
C (0,052 ± 0,008 / 4,58 ± 0,62 / 6,7 ± 4,0) und
D (0,054 ± 0,011 / 4,49 ± 0,63 / 8,3 ± 5,9).

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Mit Hilfe einer modifizierten *Scatchard*-Analyse wird gezeigt, daß statistisch signifikante Unterschiede zwischen den Receptor-Affinitäten der Gruppen A und D, B und D sowie A und C existieren.

Die Differenzen der Receptor-Affinitäten und -Konzentrationen zwischen follikulärer und lutealer Cyclusphase sind nicht signifikant. Zwischen der Insulin-Konzentration im Plasma und der Bindung des [^{125}I]Insulins an den Receptor besteht kein umgekehrt proportionaler Zusammenhang, d.h. das Phänomen der Downregulation der Receptor-Konzentration scheint für den Erythrocyten nicht zuzutreffen.

Die Resultate deuten darauf hin, daß am Erythrocyten die Bindung von Insulin bevorzugt oder ausschließlich durch eine Änderung der Receptor-Affinität moduliert wird und daß kurzzeitige Fluktuationen in der Insulin-Bindung offensichtlich nicht durch Veränderungen der Receptor-Konzentrationen bewirkt werden.

Introduction

Hormone binding to specific receptors located on the plasma membrane of a target cell is believed to be the first step in the sequence of events resulting in a definite biological response (1–3). The magnitude of this response depends on the hormone concentration, the receptor concentration and the affinity of the receptor, whereby alterations in any one of these can alter the biological response. Insulin receptors have been defined in human mononuclear cells (4–8) adipocytes (2, 5), placental cells (9, 10), cultured lymphocytes (11), hepatocytes (12), cultured fibroblasts (11), myocytes (13), granulocytes (14), reticulocytes (15) and erythrocytes (11, 16–37).

Insulin action has been studied at the cell receptor level, and the insulin binding and therefore insulin sensitivity, has been shown to be altered in a number of pathological states (2, 4–7, 9, 12, 13, 16, 20, 27, 31, 38–42). The insulin receptor characteristics of the human erythrocytes were shown to be similar to those of other tissue cells (1, 2, 8, 10, 12, 13, 16, 20, 24). Parallel alterations of insulin receptors of cells of various body tissues and monocytes on one hand (40) and of monocytes and erythrocytes on the other hand (27, 31, 33, 38, 41, 42) have led to the conclusion that erythrocytes thus can be considered as representative of insulin receptors on the cells of other body tissues. Furthermore, it has been proposed that insulin binding to human erythrocytes is a general measure of the insulin sensitivity of body tissues (16, 20, 31).

Insulin binding to receptors is not a fixed biological process but is subject to modulation by alterations in either receptor number or affinity (2, 30, 43). The former was thought to occur only in long-term regulation (4, 44, 45), but recently an insulin-induced receptor loss mediated by internalization has been suggested (3) to explain a rapid downregulation of the insulin plasma membrane receptors in the presence of high hormone concentrations (46). Acute alterations in insulin binding seem to be most commonly caused by changes in receptor affinity (4, 44, 45, 47, 48).

The most popular method of graphical analysis of equilibrium binding data is the one described by *Scatchard* (49–52) to decide whether receptor concentration or affinity is altered. Binding data at equilibrium, however, do not distinguish between multiple classes of independent sites, negative cooperativity in binding or both multiple classes of cooperatively interacting sites. Until now the negative cooperativity model has been proposed for the insulin receptor (49) and it is generally used to explain the curvilinear *Scatchard* plots of insulin binding data, though in the past two years several reports have questioned this concept (53). Furthermore, from the literature one can observe a peculiar correlation between the highest ligand concentrations chosen by the investigator for extrapolation of the *Scatchard* plot to provide an abscissa intercept and the calculated number of binding sites per cell derived from this intercept (fig. 1).

There is no definite criterion to decide the highest total ligand concentration for extrapolation of the terminal slope of the *Scatchard* plot to provide the total receptor concentration. Each investigator has therefore chosen his own concentration, with the result that a variety of values for the number of receptors (between 10 and 2000/normal erythrocyte) has been described in the literature (fig. 1).

From our recent findings on the influence of the non-specific binding (54) on *Scatchard* plot data, we decided to use the initial part of the *Scatchard* plot only for the calculation of binding parameters.

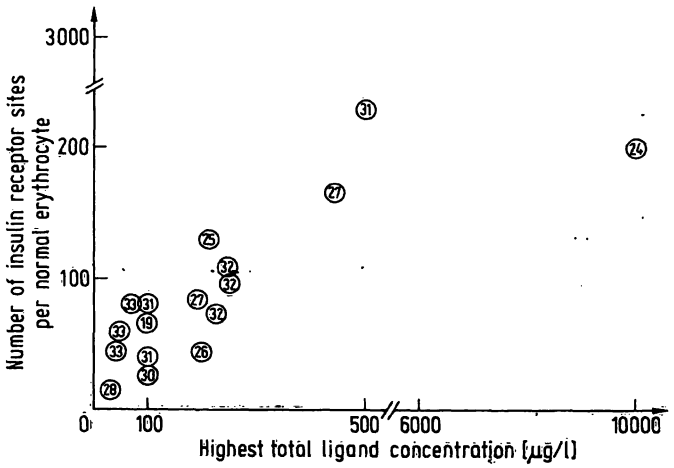

Fig. 1. Number of insulin receptor sites per erythrocyte (ordinate) and highest total ligand concentration (abscissa) used to provide the maximal amount of insulin bound according to the *Scatchard* plot. Each circle represents data from literature; the reference numbers are enclosed.

The insulin receptor may be altered in many common situations, as well as in rare disorders, or situations accompanied by insulin resistance such as diabetes (2, 20, 32, 55), obesity (4), acromegaly (56), acanthosis nigricans (7), anorexia nervosa (27, 42), uraemia (57), ataxia telangiectasia (6), pregnancy (9, 34, 35, 58) and during the menstrual cycle (33, 59, 60).

By including the modified *Scatchard* analysis, the present study is an attempt to elucidate an inter-relationship between the known insulin resistance during pregnancy and its reflection in an altered insulin binding to erythrocyte insulin receptors, by comparing the postpartum state, the follicular and the luteal phases of the menstrual cycle.

Materials and Methods

Subjects

Four groups of nonobese healthy females were studied:

A 18 women within two weeks before delivery
B 20 women within six days after delivery
C 12 women within the follicular phase of the menstrual cycle
D 11 women within the luteal phase of the menstrual cycle.

None of the subjects were taking any drug known to affect carbohydrate or insulin metabolism and none had a family history of diabetes. About 12 ml blood were drawn into a heparinized syringe (75 000 IU/l blood) in the morning after an overnight fast by venipuncture.

Reagents and equipment

Hoechst AG: porcine [^{125}I]insulin, specific activity 6.41 GBq/mg (175 mCi/mg), lyophil.;
Novo: porcine insulin, 10 × crystallized, 1 mg = 25 IU;
Serva: bovine albumin, lyophil., pure, 92% (*Cohn*-Fraction V);
EDTA · Na_2 · H_2O;
E. Merck: NaCl; KCl; $CaCl_2$ · $2H_2O$; $MgCl_2$ · 6 H_2O; *D*(+)-glucose; Tris(hydroxymethyl)amino methane (TRIS); barbital sodium; sodium acetate, anhydrous; sodium azide; trichloroacetic acid; dibutyl phthalate, density 1.05 g/cm^3;
Sigma: HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid);
Pharmacia: Ficoll-Paque (100 ml contains: 5.7 g Ficoll 400, 9 g Diatrizoate sodium), density 1.077 ± 0.001 g/cm^3; Sephadex G-10;
Hoffmann-La Roche: Liquemin 25000 (heparin sodium salt).

Buffer 1: NaCl 50 mmol/l; KCl 5 mmol/l; $CaCl_2$ 10 mmol/l; $MgCl_2$ 10 mmol/l; TRIS 50 mmol/l; EDTA · Na_2 2 mmol/l; HEPES 50 mmol/l; *D*-glucose 10 mmol/l; bovine serum albumin 1 g/l; pH 7.4 at 15 °C;

Buffer 2: barbital sodium 7.13 mmol/l; sodium acetate 11.8 mmol/l; sodium chloride 138 mmol/l; sodium azide 15.4 mmol/l; bovine serum albumin 1 g/l; pH 7.4.

Trichloroacetic acid in water 100 g/l;
Boehringer Mannheim: Test-Combination Glucose for the determination of glucose by the glucose oxidase method;
Insulin was determined radioimmunologically as described elsewhere (61).

Erythrocyte preparations

The preparation of human erythrocytes was performed according the procedures of *Böyum* (62) and *Gambhir* (23) with slight modifications. After centrifugation of the heparinized blood samples at 800 *g* for 10 min at room temperature the plasma was aspirated, frozen and saved for the assays of insulin and glucose. The cell pellet was resuspended with two parts of physiological saline, layered on 3 ml Ficoll-Paque and centrifuged at 400 *g* for 20 min at 20 °C. The supernatant including the upper layer of the erythrocyte phase was aspirated and the cell pellet suspended in 2.5 parts of physiological saline, and the above procedure repeated. The resulting cell pellet was then suspended in 2.5 parts of buffer 1 to equilibrate the cells. After centrifugation of the cell suspension at 800 *g* for 15 min at 20 °C the buffer was aspirated and the cell pellet resuspended in buffer 1, so that the cell suspension contained 3.3–6.6 · 10^{12}/l. Cell counting was performed using a *Neubauer* chamber.

Purification of [^{125}I]insulin

The labelled hormone was purified every three days using a Sephadex G-10 column (15 × 1.4 cm) with buffer 2 at a speed of 0.5 ml/min. 0.5 ml fractions were collected and the elution pattern showed one peak at the fraction numbers 6–10 (insulin) and another at the fraction numbers 25–35 (degradation products). Fractions 6 and 7 were combined and diluted with buffer 1 to give a concentration of 0.9 nmol/l.

Insulin standard solutions

Crystallized insulin was dissolved in 0.03 mol/l HCl and diluted with buffer 1 to give various concentrations between 0.87 and 86 956 nmol/l.

Nonspecific binding and degradation of insulin

Nonspecific binding, defined as the radioactivity associated with erythrocyte pellets in the presence of an insulin concentration of 17.4 µmol/l, was subtracted from total binding to yield specific binding. Nonspecific binding (fraction of total radioactivity) was 0.0199 ± 0.0023 for group A, 0.0203 ± 0.0063 for group B, 0.0187 ± 0.0033 for group C and 0.0193 ± 0.0032 for group D.

Insulin degradation was measured by examining the ability of the unaltered [^{125}I]insulin and the B-chain to precipitate in the presence of 50 g/l trichloroacetic acid (63). The fraction of insulin degraded during incubation was calculated by

$$\text{Fraction of insulin degraded} = \frac{(\text{fraction of soluble activity in the supernatant}_{t=3\,h} - \text{fraction of soluble activity in the supernatant}_{t=0}) \cdot 1.00 \cdot 1.5}{1.00 - \text{fraction of soluble activity in the supernatant}_{t=0}}$$

The factor 1.5 accounts for the iodine ratio of A- to B-chain of 2 to 1 (64). The fraction of insulin degraded did not differ between the subject groups and averaged 0.0456 ± 0.0223.

Insulin binding studies

400 µl erythrocytes (3.3 – 6.6 · 10^{12}/l buffer 1) were incubated with 50 µl [^{125}I]insulin solution (0.9 nmol/l buffer 1) and 50 µl insulin standard solutions (0.87 – 86 956 nmol/l buffer 1) or 50 µl buffer 1 in a water bath at 15 °C with shaking (60 min^{-1}) for 3 hours. After incubation, 200 µl duplicate aliquots were transferred to prechilled Eppendorf tubes containing 250 µl of buffer 1 and 250 µl of dibutyl phthalate. The tubes were centrifuged at 12000 *g* for 1.5 min and the total radioactivity of each tube counted in a gamma counter until 10 000 counts had been accumulated. The supernatants were then aspirated with a Pasteur pipette, leaving about 50 µl of dibutyl phthalate on the pellet. The tips of the tubes were cut off with a heated scalpel and counted for 10 min.

Data analyses

The fraction of radioactivity bound was determined by

$$\text{Fraction B} = \frac{\text{erythrocyte pellet radioactivity}}{\text{total radioactivity in 200 µl of the incubated cell suspension}}$$

The fraction of specific insulin bound at each concentration of unlabelled insulin was determined by subtracting the fraction of [^{125}I]insulin bound in the presence of 17.4 µmol/l insulin from the total fraction of [^{125}I]insulin bound at each concentration of unlabelled insulin.

The amount of insulin specifically bound was normalized to a cell concentration of $4 \cdot 10^{12}$/l.

The data on steady state binding of [^{125}I]insulin to its receptor are presented in two ways that emphasize different quantitative aspects of the binding interaction: the fraction of specifically bound [^{125}I]insulin is plotted as a function of the log total insulin concentration, and the bound to free ratio (B/F) of [^{125}I]insulin is plotted as a function of bound hormone (*Scatchard* plot).

Individual *Scatchard* plots were made for each subject, the slope b of the initial part of the *Scatchard* plot ($-K_a$) was calculated by regression analysis of the first seven points up to the 'critical ligand concentration'; its intercept with the abscissa gave the corresponding maximal amount of insulin bound (x_o) from which the number of receptor sites per cell R_o was calculated by

$$R_o = \frac{\text{insulin bound (mol/l)} \cdot 6.023 \cdot 10^{23}}{\text{cell concentration (cells/l)}}$$

R_o values of groups B, C and D were normally distributed, the distribution for group A was positively skewed, but log (R_o) was normally distributed. Concerning the data of the slopes, arc tan b values were compatible with a *Gaussian* distribution. Statistical comparisons between R_o or log (R_o) values and arc tan b values, respectively, were calculated by *Mann-Whitney*'s U-test and by *Student*'s t-test.

Results and Discussion

The fasting insulin and blood glucose levels did not differ for the follicular and the luteal phases whereas, as already known (65), the insulin levels of the pregnant and the post-partum groups were higher and the blood glucose levels were lower in comparison with the nonpregnant states (tab. 1). Maximal specific [^{125}I]insulin binding was highest in group A followed by group B, whereas the fraction of binding of the groups C and D were lowest and nearly identical (tab. 2). The competition-inhibition curves for the four different groups studied are shown in figure 2. The half maximal competition concentration was read from this plot (tab. 2). The *Scatchard* plots (fig. 3) were curvilinear when considering the entire range of total ligand concentrations investigated. However, in view of our recent findings (54) and their consequences, only the initial part of the *Scatchard* plot up to 2 nmol/l was used for calculation of the affinity constants, K_a, and the total number of receptor sites, R_o.

Tab. 1. Fasting insulin and plasma glucose concentrations ($\bar{x} \pm s$) for four groups of women.

	Insulin (mU/l)	Glucose (mmol/l)
Pregnancy (group A)	29.4 ± 21.4	4.00 ± 0.58
Postpartum (group B)	41.5 ± 26.2	4.40 ± 0.75
Follicular phase (group C)	6.7 ± 4.0	4.58 ± 0.62
Luteal phase (group D)	8.3 ± 5.9	4.49 ± 0.63

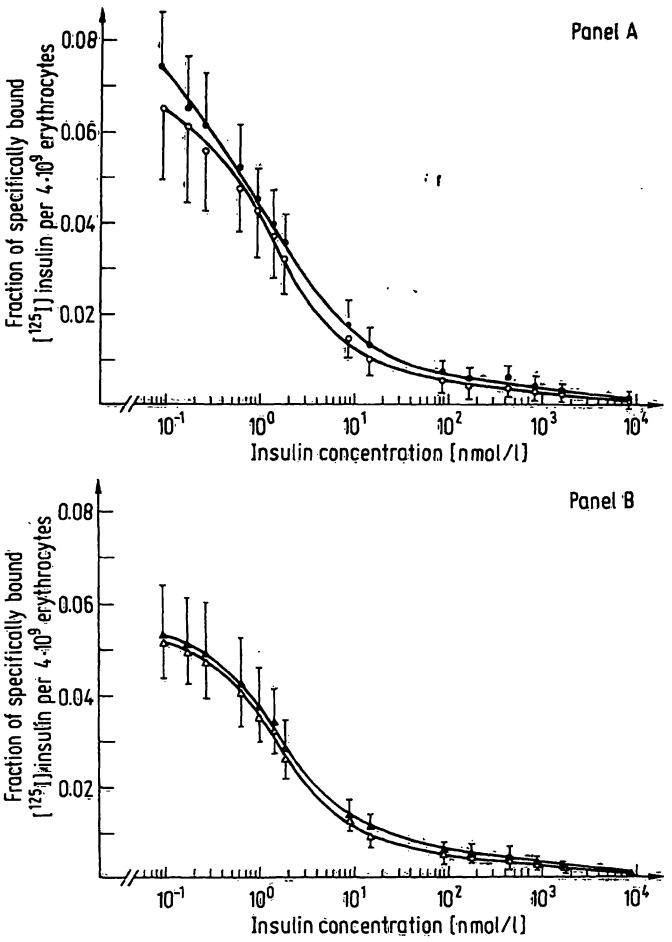

Fig. 2. Comparison of insulin binding to erythrocytes.
Panel A: pregnancy (•), postpartum state (○).
Panel B: follicular phase (△), luteal phase (▲).
Ordinate: fraction of specific [^{125}I]insulin binding ($\bar{x} \pm s$).
Abscissa: total insulin concentration (nmol/l).

By equilibrium binding studies we demonstrated that erythrocytes were saturated with specifically bound insulin in the order of 0.1–0.2 nmol/l with $4 \cdot 10^{12}$/l erythrocytes, and that up to a total ligand concentration of about 5 nmol/l and a cell count of $4 \cdot 10^{12}$/l more than 0.50 of the receptors were occupied. By further increasing of the total ligand concentrations the resulting data were more and more influenced by non-specific binding, and the terminal part of the *Scatchard* plot became artefactual and could not be used for calculation procedures. We therefore decided to use total

Tab. 2. Fraction of specific [^{125}I]insulin binding to erythrocytes ($4 \cdot 10^9$ cells) ($\bar{x} \pm s$) and half maximal competition concentration $c_{1/2}$ (nmol/l) for four different groups of women.

	Fraction of binding	$c_{1/2}$
Pregnancy (group A)	0.0741 ± 0.0123	1.50
Postpartum (group B)	0.0645 ± 0.0162	1.83
Follicular phase (group C)	0.0520 ± 0.0084	2.00
Luteal phase (group D)	0.0535 ± 0.0107	2.05

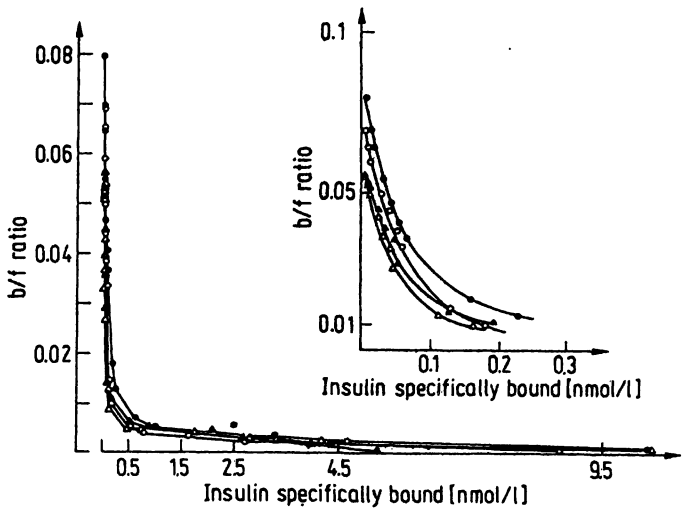

Fig. 3. *Scatchard* plots of the insulin binding data from figure 2.
Group A: pregnant state •.
Group B: postpartum state ○.
Group C: follicular phase △.
Group D: luteal phase ▲.
Ordinate: bound/free ratio.
Abscissa: insulin specifically bound (nmol/l).
Inset: initial part of the *Scatchard* plot on a larger scale.

ligand concentrations only up to 5 nmol/l with $4 \cdot 10^{12}$/l erythrocytes as a 'critical ligand concentration' for the investigation of specific insulin binding to erythrocyte receptors (54).

By focussing on the initial part of the *Scatchard* plot, curvilinearity disappeared and the character of this hormone/receptor system was reduced to one binding site with one characteristic affinity constant and a receptor concentration of one characteristic order.

The slope b ($-K_a$), the intercept with the abscissa (x_o) and the receptor number (R_o) were calculated as described above; the results are summarized in table 3, and the corresponding significances of the differences of K_a and x_o values are denoted in table 4.

The availability of reproducible methods to measure cellular receptor characteristics has facilitated efforts to understand the mechanisms of hormone action.

Tab. 3. Insulin receptor affinity ($K_a \pm s_{K_a}$), concentration ($x_o \pm s_{x_o}$) and number of binding sites per erythrocyte (R_o) for four groups of women.

	Affinity (10^8 l/mol)	Concentration (nmol/l)	Receptors/cell R_o
Pregnancy (group A)	6.68 ± 1.03	0.117 ± 0.011	18
Postpartum (group B)	6.32 ± 0.80	0.114 ± 0.031	17
Follicular phase (group C)	5.71 ± 0.70	0.101 ± 0.017	15
Luteal phase (group D)	5.28 ± 0.77	0.113 ± 0.026	17

In the case of insulin, however, two difficulties seem to prevent homogeneous results. Firstly, there has been an increasing use of red blood cells for the investigation of insulin receptor characteristics, and the erythrocyte cannot synthesize receptor proteins; therefore, it can probably be assumed that acute regulation mechanisms may be restricted to affinity modulation. However, there are several reports (33, 35, 36, 59, 60) which explain even rapid changes in insulin binding by alterations in receptor concentration evaluated by *Scatchard* analysis and by the negative cooperativity model up to high total ligand concentrations. The second uncertainty arose from the ill defined conditions in using the *Scatchard* plot to decide whether receptor concentration or affinity is altered. The higher the total ligand concentrations chosen to provide an intercept of the terminal slope with the abscissa, the higher is the calculated number of receptors per cell (fig. 1). From this observation one could conclude that the calculated number of receptor sites is limited ultimately by the solubility of the hormone.

Furthermore, there is no agreement on insulin binding to specific receptors during human pregnancy and during the menstrual cycle. *Beck-Nielsen* et al. (55) found the number of monocyte insulin receptors to be reduced during pregnancy, *Tsibris* et al. (34) reported no difference of insulin receptor number and affinity between pregnant and control groups for monocytes and for erythrocytes. *Soman* et al. (66) described an increased insulin binding to monocyte receptors during pregnancy, whereas *Moore* et al. (35) found no receptor alteration on erythrocytes during pregnancy. *De Pirro* et al. (59) reported that insulin binding to monocytes was twofold in the follicular phase compared to the luteal phase. For erythrocytes insulin binding was reported also to be higher in the follicular phase in comparison with the luteal phase. *Bertoli* et al. (33) explained this finding by a decrease of the receptor concentration and an increase of the affinity during the follicular phase, whereas no change in receptor concentration or affinity should occur during the luteal phase. On the contrary, *Moore* et al. (35) described that the decrease of insulin binding during the luteal phase is caused by a decrease in receptor number with no change in affinity. Because of these conflicting results and our previous finding of

Tab. 4. Statistical comparisons of erythrocyte insulin receptor affinities (K_a) and concentrations (x_o) for four groups of women (ns = notsignificant).

Groups		A	B	C
D	K_a	$p < 0.005$	$p < 0.01$	ns
	x_o	ns	ns	ns
C	K_a	$p < 0.05$	ns	
	x_o	ns	ns	
B	K_a	ns		
	x_o	ns		

a 'critical ligand concentration' (54) it seemed necessary to reinvestigate insulin binding to erythrocytes in the pregnant and the postpartum state in comparison with the follicular and the luteal phases of the menstrual cycle.

Pregnancy has been shown to be accompanied by insulin resistance (65, 67); the interpretation of the mechanisms underlying this interrelationship is discussed. Fasting blood glucose levels of women in the third trimester of pregnancy were found to be slightly lower than those of normal menstruating women; fasting insulin levels were found to be higher according to *Moore* et al. (35), *Tsibris* et al. (34) and *Spellacy* et al. (68). Insulin and blood glucose levels did not vary during the menstrual cycle; these results are in agreement with those of *Bertoli* et al. (33). Though the fasting insulin level of the postpartum group remained elevated within six days after delivery, the corresponding glucose level increased to concentrations comparable with those of the follicular and the luteal phases. After parturition, blood glucose levels seemed to normalize earlier than insulin levels.

Mean [^{125}I]insulin binding was slightly lower for the postpartum group in comparison to the pregnant group (tab. 2). This finding agreed with *Moore* et al. (35), but our results indicated no significant alteration, either of the receptor affinity (tab. 3 and 4) or of the receptor concentration, claimed by *Moore* et al. (35), employing the extended *Scatchard* plot and the average affinity analysis. There was no significant difference in [^{125}I]-insulin binding between the follicular and the luteal phases of the menstrual cycle (tab. 2, 3 and 4). This is in contradiction to *Bertoli* et al. (33) and *Moore* et al. (35) who reported higher insulin binding in the follicular phase and who explained this difference by changes of the receptor concentration. We suppose that this interpretation, which has been described earlier for monocytes by *De Pirro* et al. (59), may not be applicable to erythrocytes because it is poorly understood how receptors can appear and disappear (or become masked) in rhythmical two weekly fluctuations.

Tsibris et al. (34) found no difference in [^{125}I]insulin binding to erythrocytes in pregnancy and in the luteal phase of the menstrual cycle, whereas *Moore* et al. (35) reported that insulin binding to erythrocytes was highest in the follicular phase and in pregnancy and both were significantly higher than insulin binding during the luteal phase. Like *Moore* et al. (35) we found insulin binding in the pregnant group higher than that in the luteal phase (tab. 2), but our data from the modified *Scatchard* analysis indicated a significant alteration in receptor affinity, whereas *Moore* et al. (35) described a variation in receptor concentration. Furthermore we found that [^{125}I]insulin binding in the pregnant group was higher than that in the follicular phase, and this was caused by an alteration of receptor affinity. Parturition did not alter the insulin receptor characteristics; K_a and R_o values of the groups A and B were very similar (tab. 3 and 4). Despite the high insulin levels (tab. 1) groups A and B showed the highest insulin binding which was based on high affinity constants but unaltered receptor concentrations (tab. 2 and 3). We were therefore unable to find the well-known inverse correlation between insulin binding and plasma insulin concentration for erythrocytes, i.e. the well-documented downregulation of insulin receptor concentration on plasma membranes of other cell types caused by hyperinsulinaemia. Like *Tsibris* et al. (34) we found that insulin receptors did not decrease during pregnancy, despite hyperinsulinaemia, and suggest that some factors exist during pregnancy that modulate insulin receptors in a positive manner, as seen by an increased affinity and partially by an increased receptor concentration (tab. 3 and 4).

In summary, the current results indicate that erythrocytes seem to modulate insulin binding chiefly or exclusively by an alteration of receptor affinity, and that short-term changes of insulin binding to erythrocytes could not be explained by an alteration of receptor concentration.

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